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THE ABSENCE OF DEMONSTRABLE SPECIFIC ANTIBODIES IN RABIES CAUSED BY FIXED VIRUS *

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Our inability to isolate, grow, and identify microscopically the causal agent in rabies has rendered the study of its biologic reactions doubly difficult. A great number of organisms have been described. (Noguchi[1] has recently described an organism with which he has been able to produce rabies in animals.) Despite the fact that in the prevalent prophylactic treatment in rabies we are able to attain an immunity, or better a resistance, of an exceedingly high degree, specific serum reactions, especially reactions of diagnostic value, are as yet unknown. The mechanism of the immunity and the factors which produce this immunity have not been definitely determined so as to be generally accepted.

Babes[2] asserted that he had produced immunity by inoculating dogs with normal brain tissue. Fermi[3] and Repetto[4] believed that animals treated in this way possessed a greater immunity than animals immunized with rabies brains. Fermi[5] found further that fresh egg-yolks and mixtures of cholesterin, and lecithin were capable of lending animals an immunity to rabies. That the increased resistance obtained in this way is non-specific must be concluded. These experiments, however, lay open to doubt the specific immunity as supposedly obtained in the routine Pasteur treatment or in any of its many modifications.

The passage of street virus through rabbits so reduces the virulence for man that this material when inoculated into the latter is readily destroyed, presumably before the virus reaches the central nervous system. It has been assumed that in this way the formation of specific antibodies is stimulated upon which the subsequent immunity is dependent. It would seem plausible that a specific rabicidal serum could be produced. Kraus, Keller, and Clairmont[6] were able to prove that the serum of immunized animals was rabicidal whereas that of non-immunized animals was non-rabicidal. As early as 1889 Babes and Lepp[7] demonstrated that the serum of an immune animal was capable of rendering rabies virus non-virulent when allowed to act upon the latter in vitro. Such serum

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1. Presse méd., 1913, 21, p. 729; Berl. klin. Wchnschr, 1913, 50, p. 1931.
2. Cited, Kolle and Wassermann, Handb. d. Pathogen. Mikroorganismen, 1913, 8, p. 902.
3. Centralbl. f. Bakteriol., I, O., 1907, 44, p. 475.
4. Ibid., 1909, 51, p. 581.
5. Ibid., 1908, 48, p. 357.
6. Ztschr. f. Hyg. u. Infections-krankh., 1902, 41, p. 486.
7. Ann. de l'Inst. Pasteur, 1889, 3, p. 384.

has not, however, been found to lend an unquestioned passive immunity to other animals. Centanni[8] noted that in an animal in the process of active immunization the rabicidal power of its serum was greater before full immunity was established than it was in the same animal at a later date when full immunity had been produced. It would therefore appear that even in active immunity the individual's resistance is not dependent alone upon a single type of antibody present in its serum. It has been assumed that in this later stage a histogenetic immunity has been developed in those body cells peculiarly susceptible to the action of rabies virus. These findings, apparently paradoxic in themselves, would almost forecast the ultimate fruitlessness of an attempt to obtain a reliable, specific, diagnostic serum reaction in rabies. It is probably because of this that so little work has been done along this line. Frugoni and Gargiano,[9] when immunizing animals to rabies, obtained reactions not unlike anaphylaxis. There is no definite evidence, however, indicative that the sensitization in these instances was specific for rabies virus, and the possibility of employing such a sensitized animal for the determination of the presence of rabies virus in suspected brain is hardly feasible.

Following the work of Bordet and Gengou on complement-binding reactions, it was hoped that a method applicable to the diagnosis of rabies had been found. The essentials of a well-adapted antigen-antibody system were thought to be present; that is, a strong antigen in the powerful rabies virus and an immune serum. Heller and Tomarkin[10] and Friedberger[11] were able to obtain an inhibition in hemolysis with this reaction when using extracts of rabies brain and serum from immunized animals. The reaction, however, was found not to be specific, inasmuch as non-virus-containing brain extracts employed as antigen also were bound to complement by immune serum. On the other hand, Baroni, Cinca, Ionescu-Mihaiesti,[12] Donati and Satta,[13] and Berry and Mann[14] found no evidence of antibody formation in rabies immune serum by complement-fixation tests. Dobrowolskaja[15] got a definite inhibition in hemolysis in his experiments but found that sera from non-immune animals which were bled at the height of digestion were likewise capable of binding complement with his antigens. Bertarelli,[16] who immunized rabbits to fixed virus, asserted that he had obtained a specific complement-binding which was not obtained in his control experiments. The direct variance in the results of these different investigators suggested the possibility that some of the non-specific reactions were due in part, not to an antibody for rabies virus, but rather to an antibody for the brain tissue with which the animals were injected at the time of immunization because of our inability to separate the brain tissue from its contained virus. With this in mind, Nedrigailoff and Sawtschenko[17] prepared antigen from salivary glands of street virus animals and affirmed that they had obtained specific complement fixation with immune serum.

In our present method of immunizing animals, so much brain tissue is injected that apparently the overproduction of antibrain-tissue bodies completely masks any specific antirabies-virus bodies present. With this in mind, I determined to ascertain whether or not any specific antisubstances could be found in the serum of animals inoculated with a single lethal dose of fixed virus. Here, the amount of brain tissue injected would be at a minimum, whereas the virus, which it must be assumed multiplies, would at some time prior to death have attained a relative maximum. True, the course in rabies caused by fixed virus in rabbits is so short and rabbits appear so susceptible that the outlook for obtaining either large numbers, or an early production of demonstrable antibodies did not seem encouraging. However, in some infectious diseases the production of specific antisubstances has been known to occur very early in the disease; thus, in typhoid fever, precipitins have been found even before agglutinins, which usually are found at the end of the first week.

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[8] Deutsch. med. Wchnschr., 1893, 19, p. 1061.
[9] Berl. klin. Wchnschr., 1911, 37, p. 254.
[10] Deutsch. med. Wchnschr., 1907, 33, p. 795.
[11] Wien. klin. Wchnschr., 1907, 20, p. 879.
[12] Compt. rend. Soc. de biol., 1908, 65, p. 96.
[13] Pathologica, 1908, 1, p. 96.
[14] Jour. Exper. Med., 1910, 12, p. 339.
[15] Centralbl. f. Bakteriol., I, O., 1910, 56, p. 177.
[16] Cited, Kolle and Wassermann, Handb. d. pathogen. Mikroörganismen, 1913, 8, p. 927.
[17] Ztschr. f. Immunitätsforschung, 1911, 8, p. 353.

I am indebted for my initial virus to Dr. A. Lagorio. This virus when inoculated subdurally produced the classical fixed virus paralyses almost invariably at the end of the fifth day. From the fifth to the seventh day, the rabbit frequently suffered repeated clonic convulsions, usually ending in death at the end of the seventh, or in the early part of the eighth day. During the first five days, those rabbits the brains of which had not been greatly traumatized in the inoculation appeared quite normal. They ate almost continuously, and drank considerable water. The leukocyte count increased usually daily with considerable regularity until the fifth day, when counts of 20,000 to 22,000 were obtained. During the paralytic stages, the counts made from peripheral blood frequently dropped even as low as 8,000 or 5,000. Babes has held that the leukocytes are active either directly or indirectly in combating rabies, and, inasmuch as antibody formation has been demonstrated by Hektoen[18] and others to decrease in experimentally produced leukopenia, it was decided to make successive daily tests of the sera, inasmuch as the leukocyte counts varied in a very definite curve during the course of the disease. It was determined first to learn whether any ferments identifiable by Abderhalden's dialyzing method were present at any time during the disease. Accordingly, a series of eight rabbits and controls were inoculated subdurally, the former with a minimal lethal dose of brain containing fixed virus and the latter with an equal amount of normal brain. Babes and Pitulescu[19] have noted that sera of patients receiving Pasteur treatment for rabies split brain tissue of normal and rabid rabbits equally well, according to Abderhalden's dialyzing methods, and that this serum has no splitting action on human brain tissue. Substrates were prepared, according to the method of Abderhalden for preparing placental tissue substrates,[20] from normal rabbit brain, rabbit brain containing fixed virus, normal rabbit parotid gland and from parotid glands of rabbits afflicted with fixed virus rabies. Because of the great abundance of lipoids in brain tissue, it was also decided to learn whether fixed virus serum was more active on the substrates mentioned minus their alcohol and alcohol-ether soluble substances.

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[18] Tr. Chicago Path. Soc., 1915, 9.

[19] Compt. rend. Soc. de biol., 1914, 76, p. 207.

The substrates were extracted with absolute alcohol and then with equal parts of alcohol and ether in an incubator for several days. (Previously, substrates had been boiled until they were free from water-soluble, ninhydrin-reacting. substances.) About 1 gm. of the substrate was placed in a dialyzing sac and covered with 1.5 c.c. of serum. The sac and its contents were then placed in a receptacle containing 20 c.c. of distilled water and the water and the sac's contents covered with a layer of toluene and placed in an incubator for twenty-four hours. The dialyzing sacs were prepared from a thin 6 to 7 percent solution of celloidin in a mixture of equal parts of alcohol and ether. Only such celloidin was used as would permit a dialysis of a solution of silk peptone and would retain albumins, as evidenced by a failure of dialysis of ninhydrin-reacting substances from normal blood serum.

From a study of Table 1, it is seen that both the sera of the rabies animals and those of their controls are capable of splitting normal brain substrates as early as the second day; and that this splitting power is slightly greater in the rabies serum than in the normal serum. In most instances, somewhat before the onset of paralysis, about the fifth day of the disease, the splitting action of rabies serum is almost completely lost for all substrates. Another factor observed in Table 1 is that in almost all instances substrates of rabies brain are more easily split by both the normal serum and that of the rabid animal. The substrates of normal and rabies parotid tissue are occasionally acted upon by the rabid, as well as the control serum but only rarely is this true and then to a far less degree.

In all instances, the same substrates extracted previously with alcohol and ether are also attacked to a less degree. It would appear that this extraction breaks up some complex fat-protein molecules which in the former substrates are split by the serum with the liberation of their amino-acid constituents.

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[20] Abderhalden's Handb. d. biochem. Arbeitsmethoden, 1912, 6, p. 226.

TABLE 1

NINHYDRIN REACTION. PROTEOLYTIC POWER OF RABIES AND CONTROL SERA FOR SUBSTRATES OF NORMAL BRAIN, RABIES BRAIN, NORMAL PAROTID, AND RABIES PAROTID

Duration, in Days, of Rabies and Controls. Dialysis of Serum Alone	Normal Serum on Normal Brain Substrate	Rabies Serum on Normal Brain Substrate	Normal Serum on Rabies Brain Substrate	Rabies Serum on Rabies Brain Substrate	Normal Serum on Normal Parotid Substrate	Rabies Serum on Normal Parotid Substrate	Normal Serum on Rabies Parotid Substrate	Rabies Serum on Rabies Parotid Substrate
1—N —.........	—	—	—	++	—	+	—	+
R —.........	....	×—	....	×—	....	....	....	....
2	++	+	+	+	++	+	+	+
3—N —.........	—	+	+	+	—	—	—	—
R —.........	×—	×—	×—	×+	....	....	....	....
4—N —.........	+	++	+	+	+	—	+	—
R —.........	×+	×+	×+	....	×+	×—	×+	×—
5—N —.........	+	—	—	—	—	—	—	—
R —.........	×—	×—	×+	×—	×—	×—	×—	×—
6—N —.........	—	—	+	+	—	—	—	—
R —.........	×—	×—	×—	×+	×—	×+	×—	×+
7—N —.........	+	—	+	+	—	—	+	—
R —.........	×—	×—	×++	×—	×—	×—	×—	×—
8—N —.........	—	—	+++	++	—	—	+	++
R —.........	×—	×—	×+	×+	×—	×—	×+	×—

× = action on same substrate minus its alcohol and ether extracts.
N = normal serum.
R = rabies serum.

It was next determined to attempt to adapt the miostagmin reaction of Ascoli[21] to the identification of any reaction between antigen and antibody in rabies caused by fixed virus.

A series of 21 rabbits was inoculated subdurally with brain containing fixed virus, and controls with normal rabbit brain tissue, as in the previous series. (The sera of 8 of these rabbits were sera from the previous series.) Of this series, 8 were bled, one each on successive days following the inoculation, 4 on the fifth day just previous to the onset of paralysis and at a time when their leukocyte counts were highest, 4 on the sixth day, 7 on the seventh day, and 2 on the eighth day. Test and control antigens had been previously prepared by grinding separately rabies brain, normal rabbit brain, and parotid gland from normal and rabid rabbits. These were desiccated rapidly at 37 C. and then extracted with absolute methyl alcohol in the proportion of 1:4 at 37 C. with frequent shaking for seventy-two hours. These extracts while still warm were filtered through Scheicher and Schüll filters, No. 590, and after cooling refiltered. The extracts in various dilutions were titrated with normal rabbit sera (diluted 1:20) until a mixture was obtained that would not cause a decrease in surface tension measurable by more than one drop as determined by a Traube's stalagmometer.[22] It was found possible to use dilutions of antigen as concentrated as 1:20. The diluted serum and diluted antigen were mixed in the proportion of 9:1 and the reaction completed by keeping the mixtures at 50 C. in a water bath for one hour. The surface tension was measured by a Traube's stalagmometer by determining the difference in the number of drops for a definite volume of the serum-antigen mixtures after and before heating. The graduations on my stalagmometer are such that one drop of the serum dilutions, in general, averages 12 to 13 divisions of the scale. In this way, serum from animals at different periods in the course of rabies and from like controls were tested with extracts of normal and rabid brains and with extracts of parotid glands from normal and rabid animals (fixed virus) as seen in Table 2.

Ascoli, in applying this reaction in the diagnosis of various conditions as typhoid, carcinoma, etc., considers only such reactions positive as show an increase of two drops or more in the end reaction. From Table 2, it is seen that the controls in no instance reacted positively according to Ascoli's standard. Of the twenty-one rabies sera, only three reacted positively with rabies brain antigen, and, of these three, one reacted more strongly with normal brain antigen. One rabies serum reacted positively with normal brain antigen and failed to produce a like reaction with rabies brain antigen. Of these positive reactions, one occurred on the first day, the others on the seventh or eighth day. Parotid gland antigens failed, in the few instances in which they were utilized, to result in positive reactions.

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[21] München. med. Wchnschr., 1910, 57, p. 403.

[22] Ascoli u. Izar, München. med. Wchnschr., 1910, 57, p. 1170, p. 2129; Abderhalden's Handb. d. biochem. Arbeitsmethoden, 1912, 5, p. 1357.

TABLE 2

MIOSTAGMIN REACTION

Duration of Rabies or Control in Days	Normal Serum and Normal Brain Antigen	Rabies Serum and Normal Brain Antigen	Normal Serum and Rabies Brain Antigen	Rabies Serum and Rabies Brain Antigen	Normal Serum and Normal Parotid Antigen	Rabies Serum and Normal Parotid Antigen	Normal Serum and Rabies Parotid Antigen	Rabies Serum and Rabies Parotid Antigen
1...............	$+0^{9}$ gtt.	$+1^{1}$ gtt.	$+1^{1}$ gtt.	$+2^{2}$ gtt.	....	....	....	....
2...............	$+0^{5}$ gtt.	? gtt.	$+0^{2}$ gtt.	$+0^{8}$ gtt.	....	....	....	....
3...............	$+0^{9}$ gtt.	$+0^{5}$ gtt.	$+1$ gtt.	$+1^{2}$ gtt.	....	....	....	....
4...............	-0^{6} gtt.	$+0^{3}$ gtt.	-0^{1} gtt.	$+0^{3}$ gtt.	....	....	....	....
5...............	$+0^{5}$ gtt.	$+0^{5}$ gtt.	$+0^{2}$ gtt.	$+0^{4}$ gtt.	....	....	....	....
5...............	$+0^{2}$ gtt.	$+0^{4}$ gtt.	$+0^{4}$ gtt.	$+0^{4}$ gtt.	....	....	....	....
5...............	$+0^{2}$ gtt.	$+0^{2}$ gtt.	$+0^{4}$ gtt.	$+0^{8}$ gtt.	....	....	....	....
5...............	$+0^{2}$ gtt.	$+0^{1}$ gtt.	$+0^{4}$ gtt.	$+0^{1}$ gtt.	....	....	....	....
6...............	$+1$ gtt.	$+0^{10}$ gtt.	$+0^{8}$ gtt.	± 0 gtt.	....	....	....	....
6...............	$+1$ gtt.	$+0^{4}$ gtt. B. $+0^{1}$ gtt.	$+0^{8}$ gtt.	$+0^{1}$ gtt. B. $+0^{2}$ gtt.	....	....	....	....
6...............	$+1$ gtt.	$+0^{2}$ gtt. B. $+0^{5}$ gtt.	$+0^{8}$ gtt.	$+0^{1}$ gtt. +B. 0^{5} gtt.	....	....	....	....
6...............	$+1$ gtt.	$+0^{9}$ gtt. +B. 0^{8} gtt.	$+0^{8}$ gtt.	$+0^{11}$ gtt. B. $+0^{7}$ gtt.	....	....	....	....
7...............	$+0^{5}$ gtt.	$+0^{7}$ gtt. B. $+0^{6}$ gtt.	$+0^{5}$ gtt.	$+0^{6}$ gtt. B. $+0^{3}$ gtt.	$+0^{5}$ gtt. (C)	?	$+0^{5}$ gtt. (C)	$+0^{1}$ gtt. (C)
7...............	-0^{6} gtt.	$+1$ gtt.	-0^{1} gtt.	$+2^{1}$ gtt.	....	....	....	....
7...............	$+0^{4}$ gtt.	$+0^{5}$ gtt.	$+0^{2}$ gtt.	$+0^{4}$ gtt.	$+0^{5}$ gtt.	$+1^{4}$ gtt.	$+0^{6}$ gtt.	$+1$ gtt.
7...............	$+0^{4}$ gtt.	$+2^{12}$ gtt.	$+0^{2}$ gtt.	$+1$ gtt.	$+0^{5}$ gtt.	$+0^{6}$ gtt.	$+0^{6}$ gtt.	$+0^{7}$ gtt.
7...............	$+0^{3}$ gtt. B $+0^{11}$ gtt.	$+1^{4}$ gtt. B. $+1^{8}$ gtt.	$+0^{8}$ gtt. B. $+0^{2}$ gtt.	$+1$ gtt. B. $+1^{0}$ gtt.	(C). $+0^{3}$ gtt.	....	(C) $+0^{3}$ gtt.	....
7...............	$+0^{2}$ gtt.	$+0^{4}$ gtt.	$+0^{7}$ gtt.	$+0^{1}$ gtt.	....	....	....	....
7...............	$+0^{2}$ gtt.	$+0^{2}$ gtt.	$+0^{7}$ gtt.	$+0^{1}$ gtt.	....	....	....	....
8...............	$+0^{6}$ gtt.	$+3^{0}$ gtt.	$+0^{6}$ gtt.	$+2^{8}$ gtt.	....	....	....	....
8...............	$+0^{2}$ gtt.	$+0^{4}$ gtt.	$+0^{8}$ gtt.	$+0^{7}$ gtt.	....	$+0^{1}$ gtt.	....	$+0^{2}$ gtt.

$+1$ = increase of 1 drop; $+1^{8}$ = increase $1\frac{8}{12}$ drops; $+0^{8}$ = increase $\frac{8}{12}$ drops; etc. Antigens diluted 1:100, except B, 1:20 and C, 1:50

Inasmuch as these methyl alcohol "antigens" were rich in lipoids, it was thought that possibly some evidence of fat-splitting might be obtained with the same serum-antigen mixtures used in the miostagmin reactions. Accordingly, 5 c.c. of each mixture were titrated with N/50 NaOH and a phenolphthalein indicator before and after heating. In not one instance was the acidity after heating greater to a degree significant of lipoid-splitting, as seen in Table 3. In practically all instances, the acidity after heating for an hour was less than that of the mixture before heating. This, no doubt, was due to the expulsion of contained CO_2 in the original mixtures. In two instances, in sera of the eight-day rabies, the acidity was slightly greater in solutions of rabies sera and rabies brain. These two single instances, however, are insufficient, as compared with the great number of negative results, to warrant any general deduction.

TABLE 3

SPLITTING POWER OF SERA ON ALCOHOL-ETHER EXTRACTS OF NORMAL BRAIN AND RABIES BRAIN. TITRATIONS MADE WITH N/50 NaOH

Duration, in Days, of Rabies or Controls	Normal Serum and Normal Brain	Rabies Serum and Normal Brain	Normal Serum and Rabies Brain	Rabies Serum and Rabies Brain
1	— 0.03 c.c.	± 0.00 c.c.	+ 0.01 c.c.	± 0.00 c.c.
2	— 0.01 c.c.	— 0.02 c.c.	— 0.01 c.c.	— 0.03 c.c.
3	— 0.02 c.c.	— 0.02 c.c.	— 0.05 c.c.	— 0.03 c.c.
4	± 0.00 c.c.	— 0.04 c.c.	— 0.01 c.c.	± 0.00 c.c.
5	— 0.05 c.c.	— 0.04 c.c.	± 0.00 c.c.	— 0.02 c.c.
6	± 0.00 c.c.	± 0.00 c.c.	— 0.03 c.c.	— 0.02 c.c.
7	— 0.03 c.c.	— 0.02 c.c.	— 0.02 c.c.	— 0.01 c.c.
8	— 0.10 c.c.	— 0.05 c.c.	— 0.10 c.c.	+ 0.05 c.c.
8	± 0.00 c.c.	— 0.02 c.c.	± 0.00 c.c.	+ 0.06 c.c.

Inasmuch as precipitins are occasionally found early in the course of some infectious diseases, it was determined to learn whether any specific precipitin formation could be detected in rabies caused by fixed virus.

Precipitogen solutions were attempted by making extracts of rabies brain (1:10) with 0.85 percent NaCl solution. The brain was ground in a mortar and allowed to remain a few hours at 37 C. and then placed at +2 C. for twenty-four hours, after which it was filtered by forced aspiration through a Gouch filter. Similarly, control solutions of normal brain were prepared. A series of six rabbits was inoculated subdurally with fixed virus and controls with normal brain tissue. Three rabbits and their controls were bled at the end of the fifth day, at which in most cases their leukocyte counts ranged from 18,000 to 25,000; the white cell counts of the controls were normal. Two of the remaining series and their controls were bled at the end of the seventh, and the one remaining rabbit and control on the eighth day. The presumed precipitogen-containing extracts of both normal and rabies brain were then diluted 1 : 2 progressively in a series of twelve tubes — a procedure giving an ultimate dilution of 1 : 1248. To a tube containing 0.5 c.c. of each of these progressive dilutions were added 0.2 c.c. of serum. In this way, serum of normal rabbit was tested with extract of rabid, and extract of normal brain, respectively, and likewise sera of rabies animals at the fifth, seventh, and eighth days of the disease were tested with the similar dilutions of the same extracts for precipitins. The tubes were incubated at 37 C. for an hour and allowed to stand twenty-four hours.

In no one instance could any evidence of precipitin formation be found.

CONCLUSIONS

Protective proteolytic ferments are occasionally found at different periods of rabies caused by fixed virus. While these are at times greater for virus-containing brain tissue, they are in no sense specific for the contained virus alone. Apparently no specific splitting of virus protein can be demonstrated in substrates of the parotid gland in rabies caused by fixed virus. This may be due entirely to the absence of virus in the gland, due, in turn, to the acute duration of the disease, which does not permit the virus sufficient time to extend to the gland, as it does in street virus rabies.

Specific lipolytic ferments do not appear to be contained in the serum of rabies of fixed virus.

No evidence of antibody formation can be determined in the rabies of fixed virus by the miostagmin reaction.

There is no formation of specific precipitins in rabbits with acute rabies caused by fixed virus.